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Germination and Morphology of Spores of *Trichipteris corcovadensis*

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Several papers have dealt with the physiology of germination of pteridophyte spores. Published data show that spores of the majority of fern spores are light-sensitive. They also germinate in a wide range of temperatures, from 1°C (*Pteridium aquilinum*) to 35°C in the case of *Onoclea sensibilis* (Miller, 1968).

To our knowledge, no research has been published in Brazil on the physiology of ferns, with the exception of the germination of spores of *Cyathea delgadii* (Marcondes-Ferreira & Felippe, 1984). In this paper a study was made of germination of *Trichipteris corcovadensis* (Raddi) Copel. This species is a tree fern that occurs in the Serra do Mar area of Southern Brazil (Tryon, 1970), at elevations from 250 to 2100 meters (Barrington, 1978). A study of the spore morphology of several specimens was also carried out. Spore morphology of the species has been studied previously as *Trichopteris corcovadensis* (Kremp & Kawasaki, 1969; Erdtman & Sorsa, 1971).

MATERIALS AND METHODS

Material of *Trichipteris corcovadensis* was collected in the Reserva Biológica do Parque Estadual das Fontes do Ipiranga, São Paulo, Brazil, and identified by Dr. Paulo Windish (UNESP, São José do Rio Preto). Spores were collected from five different specimens gathered in March of different years. These collections were designated: A (1982 and 1983); B (1980, 1981, and 1982); C (1982 and 1983); D (1982); and E (1982). The spores were stored in closed bottles in the dark at 4°C. Some material from specimen B was also stored at 25°C. Spores from the five specimens were always stored separately, and the experiments were carried out with all five specimens for comparison.

Germination.—Germination studies were carried out using 25 ml of Mohr's solution, as modified by Dyer (1979), in an Erlenmeyer flask. The flasks with medium were autoclaved at 120°C for 20 minutes, and nystatin (50 units·ml^{-1}) was then added as recommended by Dyer (1979). Spores were scattered on the surface of the medium with the aid of a spatula. For each treatment, three flasks were used, and three microscope slides were prepared from aliquots from each flask. Five fields from each slide were examined through a microscope. Germination was considered as the protrusion of the rhizoid.

The experiments were carried out in growth cabinets (Forma Scientific model

24). Temperature was maintained at 25°C and light irradiance at 320 $\mu W \cdot cm^{-2}$ (continuous white fluorescent light). In some experiments the effect of constant temperature was examined (5, 10, 15, 20, 25, 30, 35, 40, and 45°C) as well as alternate temperatures of 5–25, 10–25, 15–25, 20–25, 30–25, 35–25, 40–25, and 45–25 (12 hours at each temperature in a 24 hour cycle, starting temperature 25°C). The effect of photoperiod was also studied and photoperiods of 1, 8, 12, 16 and 24 hours were used. The effect of irradiance was studied using light irradiances of 220, 320, 900, 1400, 1900, and 2500 $\mu W \cdot cm^{-2}$. The growth regulators gibberellic acid (GA_3), indole-3-acetic acid (IAA), 6-benzyladenine (6-BA) and 2-chloroethylphosfonic acid (CEPA) were used at the following concentrations: 0, 5, 25, 50, and 100 $\mu g \cdot ml^{-1}$ (the growth regulators were added to the flasks after autoclaving).

Viability of the spores was tested with acetocarmine according to Manton (1950). Spores were placed on a microscope slide with a drop of acetocarmine, covered with a cover slip, and pressed to break the spore walls (acetocarmine did not penetrate walls of intact spores). Spores with broken walls were counted 36 hours later, whether stained or not (data are presented as percentage of stained spores in relation to total spores with broken walls; 15 replicates were used).

Germination data are presented as the angular value: arc sin $\sqrt{\%}$. The percentage value is the number of germinated spores in relation to the total number of spores in each field. Statistical analysis was performed with data transformed into angular values. Where necessary the confidence interval (at 95%) and coefficient of variation are presented. Analysis of variance was also performed, and when $F_{5\%}$ was significant the $LSD_{5\%}$ was determined by Tukey's method as modified by Snedecor (1962). Lower case letters in tables compare the values within columns, taking into account the $LSD_{5\%}$.

Lipids and protein.—Total lipids were determined according to Gemmrich (1977), using 50 mg of spores in each extraction. Each specimen was analyzed in triplicate. Data are presented as mg lipid·100 mg^{-1} of spores. Soluble protein was determined according the dye-binding method of Bradford (1976), using 200 mg of spores in each determination and three replicates per treatment. Data are presented as mg protein·100 mg^{-1} spores.

Morphology of spores.—The spores were submitted to acetolysis according to Erdtman (1969). To observe the perine, spores were treated with sodium carbonate at 3% before acetolysis (Morbelli, 1974). The Wodehouse (1935) method was used to determine the presence of cellular content in the spores.

At least five slides were prepared for each sample. Measurements were made with 25 acetolysed spores, distributed on at least three slides (Salgado-Labouriau et al., 1965). Spores were measured not later than seven days from the preparation of the slide, to avoid swelling of spores (Melhem & Matos, 1972). The percentage of different spore shapes was determined in 45 fields, distributed on at least three slides (the same for the observation of the perine). To obtain the photomicrographs, an Olympus Vanox photomicroscope with an automatic camera was used. Kodak Panatomic-X, was used with a green filter to obtain details of the spores.

For specific details, the scanning microscope was also used, as described by

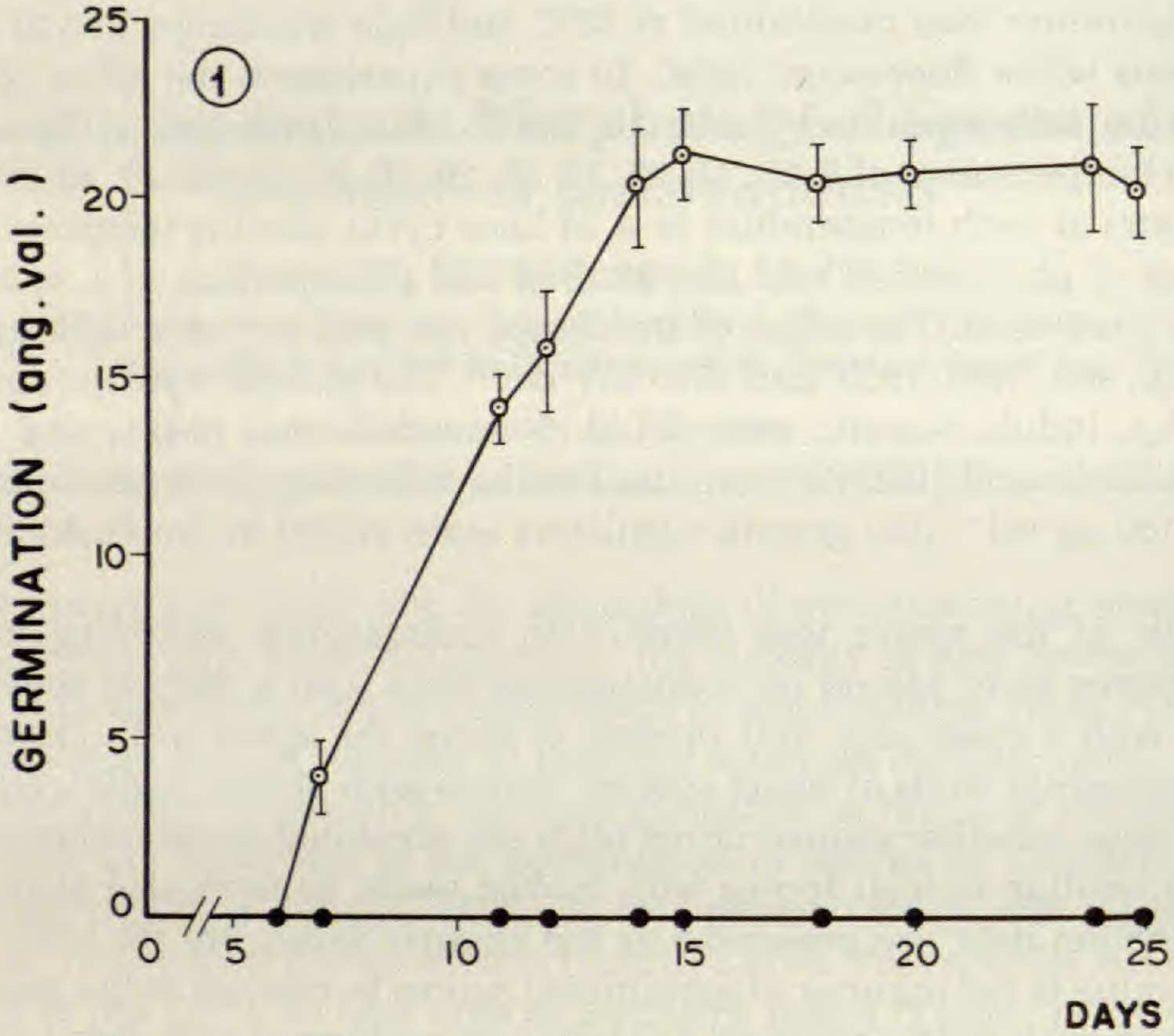


FIG. 1. Germination of spores of specimen A of *Trichipteris corcovadensis* at 25°C in constant white fluorescent light (320 $\mu W \cdot cm^2$) and darkness over a 25 day period. Open circles: light; closed circles: darkness. Confidence intervals (5%) are shown.

Cruz & Melhem (1984). Photographs were obtained from a scanning electron microscope GO, model JSM P15, with Kodak PX-135 (ASA 100 film).

The measurements presented were subjected to an analysis of variance, and when $F_{5\%}$ was significant, the $LSD_{5\%}$ (Tukey) was determined (Snedecor, 1962).

TABLE 1. Effect of Temperature on the Germination of Spores of *T. corcovadensis* (Specimen A) Kept in Continuous White Light.

Temperature* (°C)	Germination (angular value)
20	3.0[a]**
25	16.6[b]
30	10.4[c]
$LSD_{5\%}$ (Tukey)	1.8
25–20	12.7[a]
25–25	16.6[b]
25–30	13.0[a]
$LSD_{5\%}$ (Tukey)	2.4

* No germination occurred at 5, 10, 15, 35, 40, 45°C and in the alternate pairs 25–5, 25–10, 25–15, 25–35, 25–40 and 25–45°C.

** See Materials and Methods; a, b, and c, compare the values within columns.

TABLE 2. Effect of Light Irradiance and Photoperiod on the Germination of Spores of *T. corcovadensis* (Specimen A) Kept at 25°C.

Irradiance ($\mu W \cdot cm^{-2}$)	Germination (angular value)	Photoperiod (h)	Germination (angular value)
220	18.0[a]	0	0.0
320	18.9[a]	1	0.0
900	13.8[b]	8	17.6
1400	13.4[b]	12	18.5
1900	12.9[b]	16	17.7
2500	13.6[b]	24	18.9
$LSD_{5\%}$ (Tukey)	3.0		

$F_{5\%}$ not significant for photoperiods of 8, 12, 16, and 24 h.

RESULTS AND DISCUSSION

In all experiments dealing with germination, spores were obtained only from specimen A.

Figure 1 shows the results of one experiment in which spores were kept at 25°C either in continuous white fluorescent light or in darkness. No germination occurred in darkness and this was observed in all experiments. In the light, germination started on day 6. This proceeded at a fast rate until day 14 when a plateau was reached and maintained up to day 25. The maximum germination value was around 20 (angular value). In subsequent experiments germination was always counted 14 days after the start of the experiment.

No germination occurred at 5, 10, 15, 35, 40, and 45°C, even when these temperatures were alternated daily with 12 hours at 25°C. Germination occurred at 20, 25, and 30°C and with the alternate pairs 20–25 and 25–30°C (Table 1). At all temperatures tested, the spores germinated only in the light. Germination was higher with the two lower light irradiances used (Table 2) and germination occurred only (with the photoperiods tested) with photoperiods of 8 h and above (Table 2). In all the other experiments mentioned in this paper the light irradiance used was 320 $\mu W \cdot cm^{-2}$ and light was continuous (including the experiments in which temperature was tested shown previously).

TABLE 3. Effect of Growth Regulators on the Germination of Spores of Specimen A of *Trichipteris corcovadensis*, in Continuous Fluorescent White Light, at 25°C.

Concentration ($\mu g \cdot ml^{-1}$)	Germination (angular value)			
	IAA	GA_3	6-BA	CEPA
0	18.8[a]	18.5[a]	16.9[a]*	17.9
5	14.1[b]	17.2[a]	9.7[b]*	0.0
25	3.6[b]	10.2[b]	0.0	—
50	0.0	11.7[b]	—	—
100	—	3.3[c]	—	—
$LSD_{5\%}$ (Tukey)	2.2	1.8	—	—

* Student test.

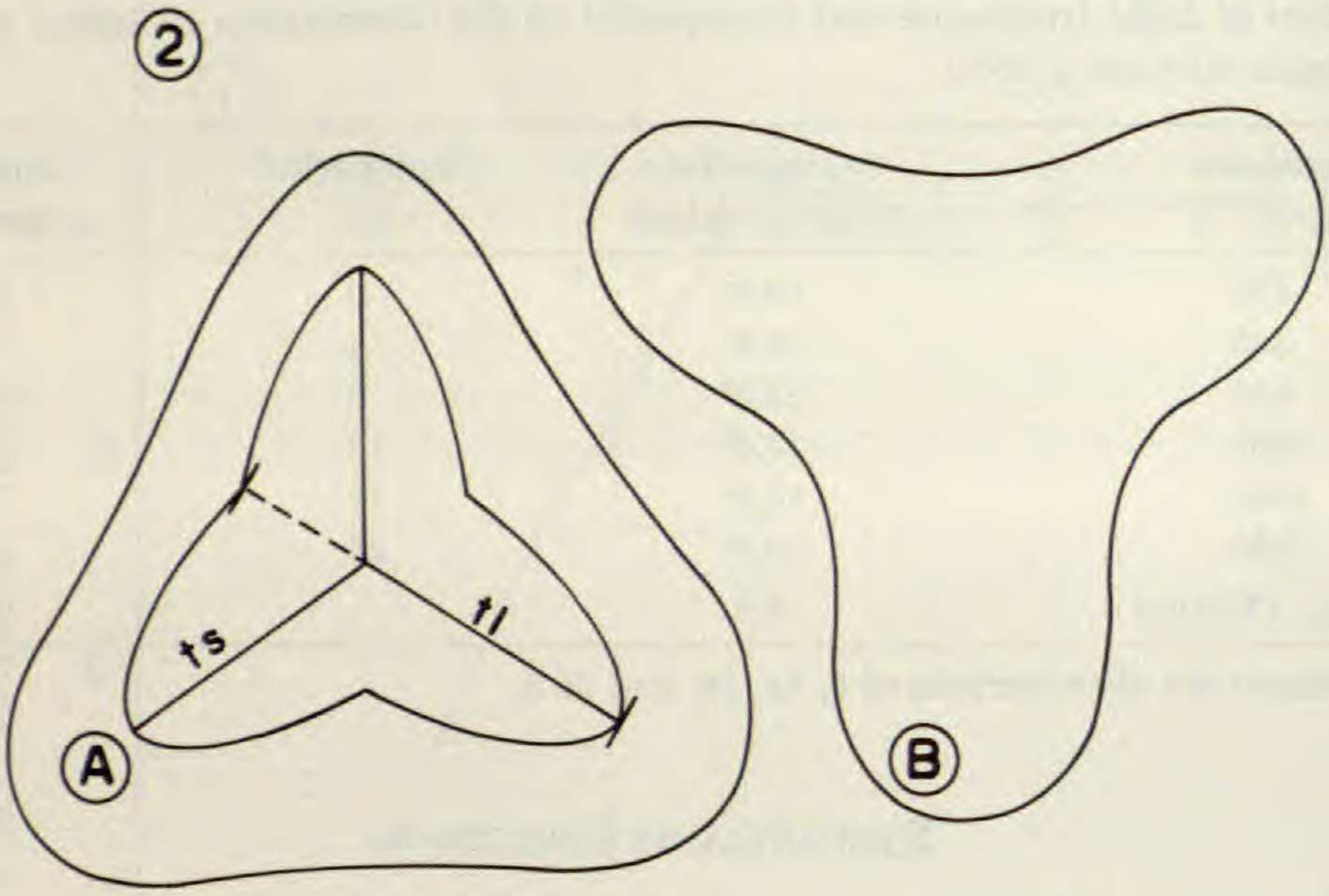


FIG. 2. Schematic representation of spores of *Trichipteris corcovadensis* showing the two extreme shapes. A = shape I; tl = trilete length; ts = trilete scar. B = shape II.

Spores of *T. corcovadensis* are positively photoblastic like the majority of pteriodophytes: Miller (1968) in his review showed that in 75 species studied only 4 germinated in darkness. *Cyathea delgadii* occurs with *T. corcovadensis* in the same region, shows germination over a wider range of temperatures (15 to 30°C), and is also a photoblastic positive species (Marcondes-Ferreira & Felippe, 1984).

All growth regulators tested had an inhibitory effect upon germination (Table 3) and were also unable to change the photoblasticity of the spores.

In all experiments the maximum values for germination were always low. This can also be seen in Table 4, in which the effect of period of storage at 4°C on germination is shown. After 135 days, germination is reduced. This was valid for spores of specimen A which were collected both in 1982 and 1983.

TABLE 4. Effect of Storage on the Germination of Spores of *Trichipteris corcovadensis* (Specimen A) in Continuous Fluorescent White Light at 25°C.

Storage at 4°C (days)	Germination (angular value)
15	19.1[a]
67	17.0[a]
92	17.1[a]
96	18.9[a]
102	18.4[a]
108	19.2[a]
134	16.9[a]
135	18.7[a]
238	11.5[b]
239	9.0[b]
254	9.2[b]
$LSD_{5\%}$ (Tukey)	2.9

TABLE 5. Shape Variation of Spores of Five Specimens of *Trichipteris corcovadensis* Collected in Different Years.

Specimen—year	Shape I	Shape II	Intermediate shapes
A—1982	100.0	0.0	0.0
B—1980	98.1	0.0	1.9
B—1981	96.4	0.0	3.6
B—1982	28.6	27.1	44.3
C—1982	30.5	22.9	44.6
D—1982	28.5	14.3	57.2
E—1982	31.5	22.4	46.1

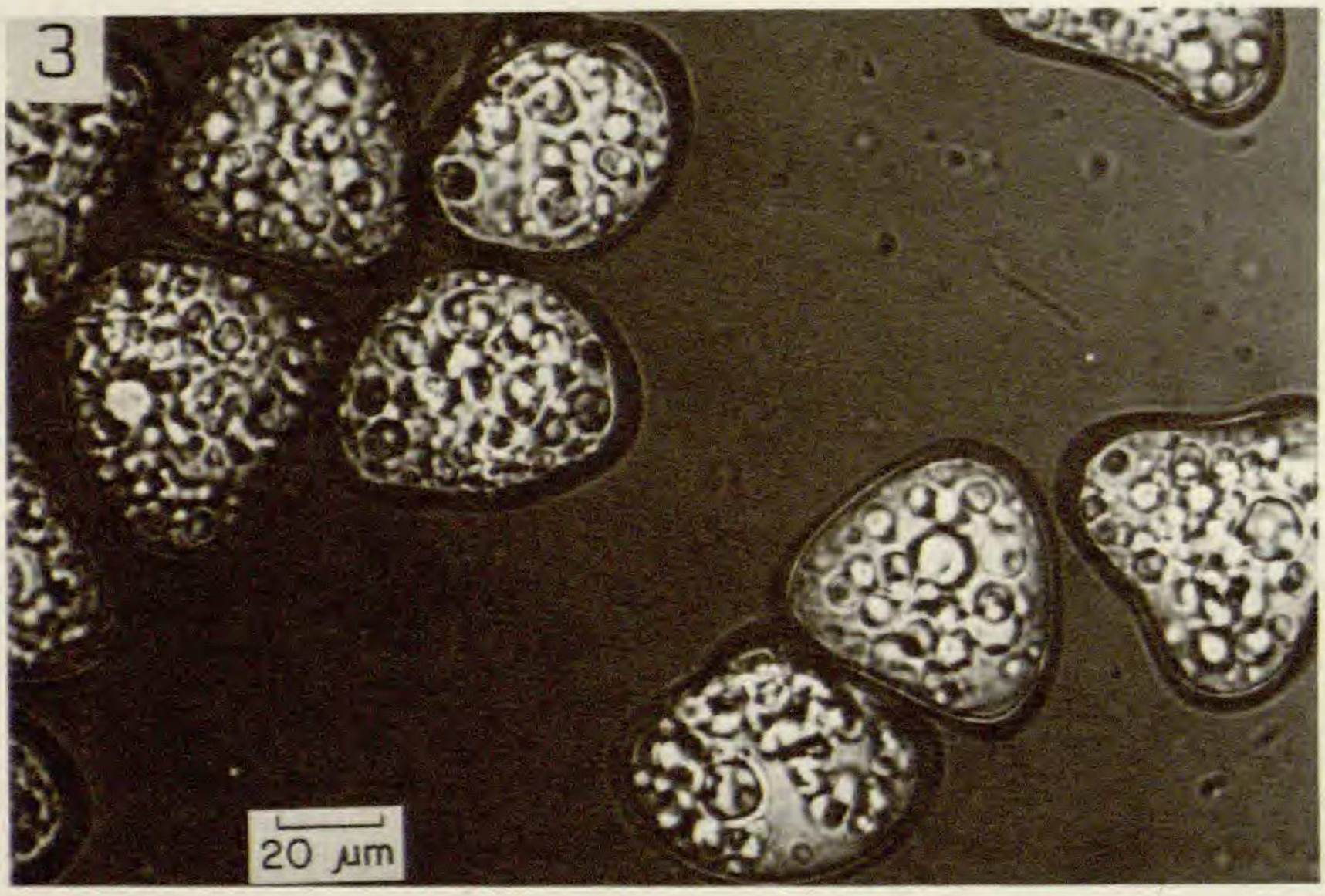
Spores collected from specimens B, C, D, and E never germinated. No treatment was able to change this. This lack of ability to germinate was observed with both freshly collected spores and stored spores. For example, spores of specimen B were stored at 4°C and 25°C and germination was tested from 15 to 960 days in regular intervals, but germination never occurred.

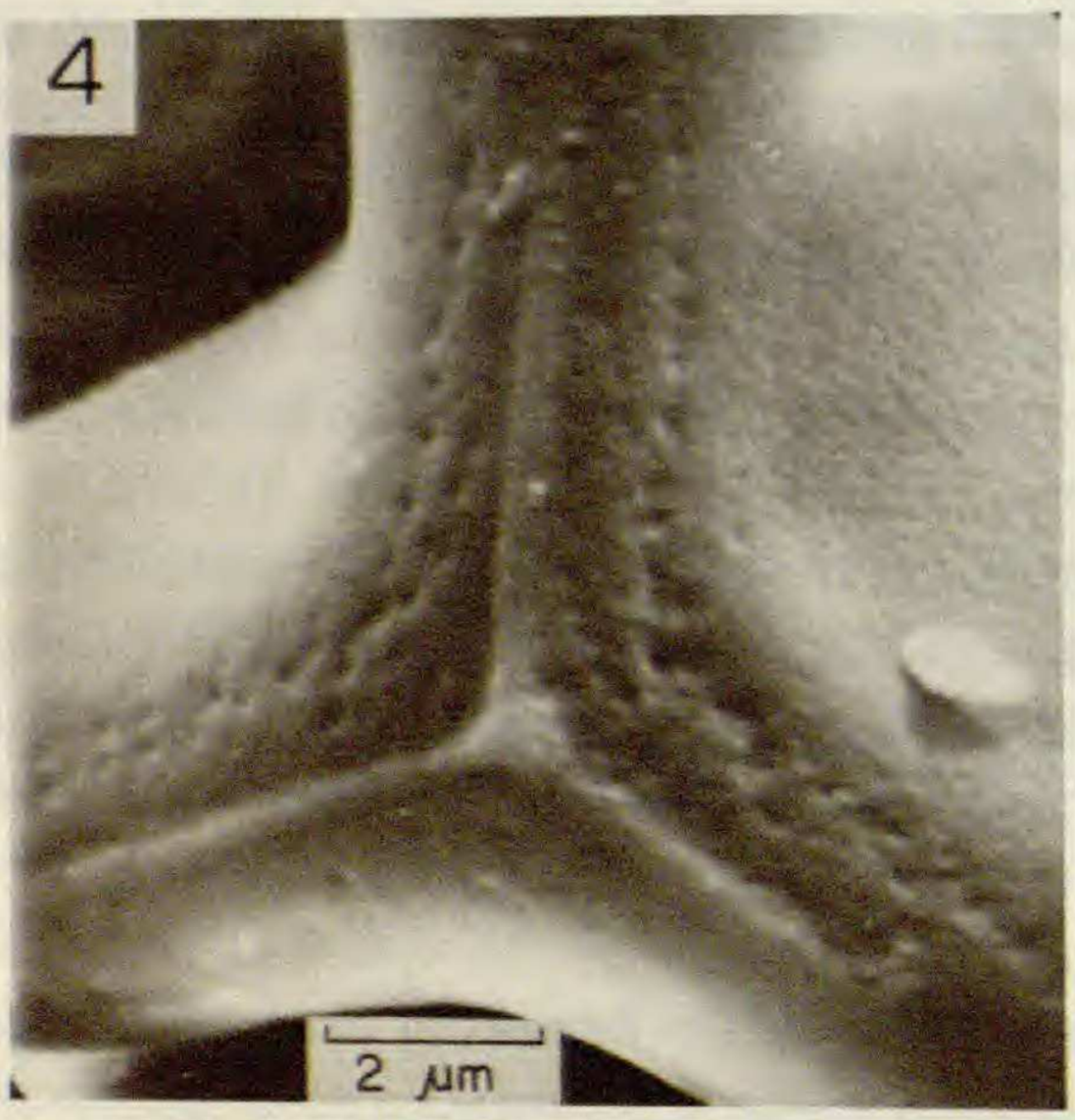
After acetolysis two extreme spore shapes could be seen: I (Fig. 2A) and II (Fig. 2B) and intermediate shapes between I and II. All spores of specimen A belonged to shape I (Table 5). Spores of specimen B collected in 1980 and 1981 belonged to shape I (around 95%) and intermediate shapes; specimens C, D, and E presented shapes I, II, and intermediates.

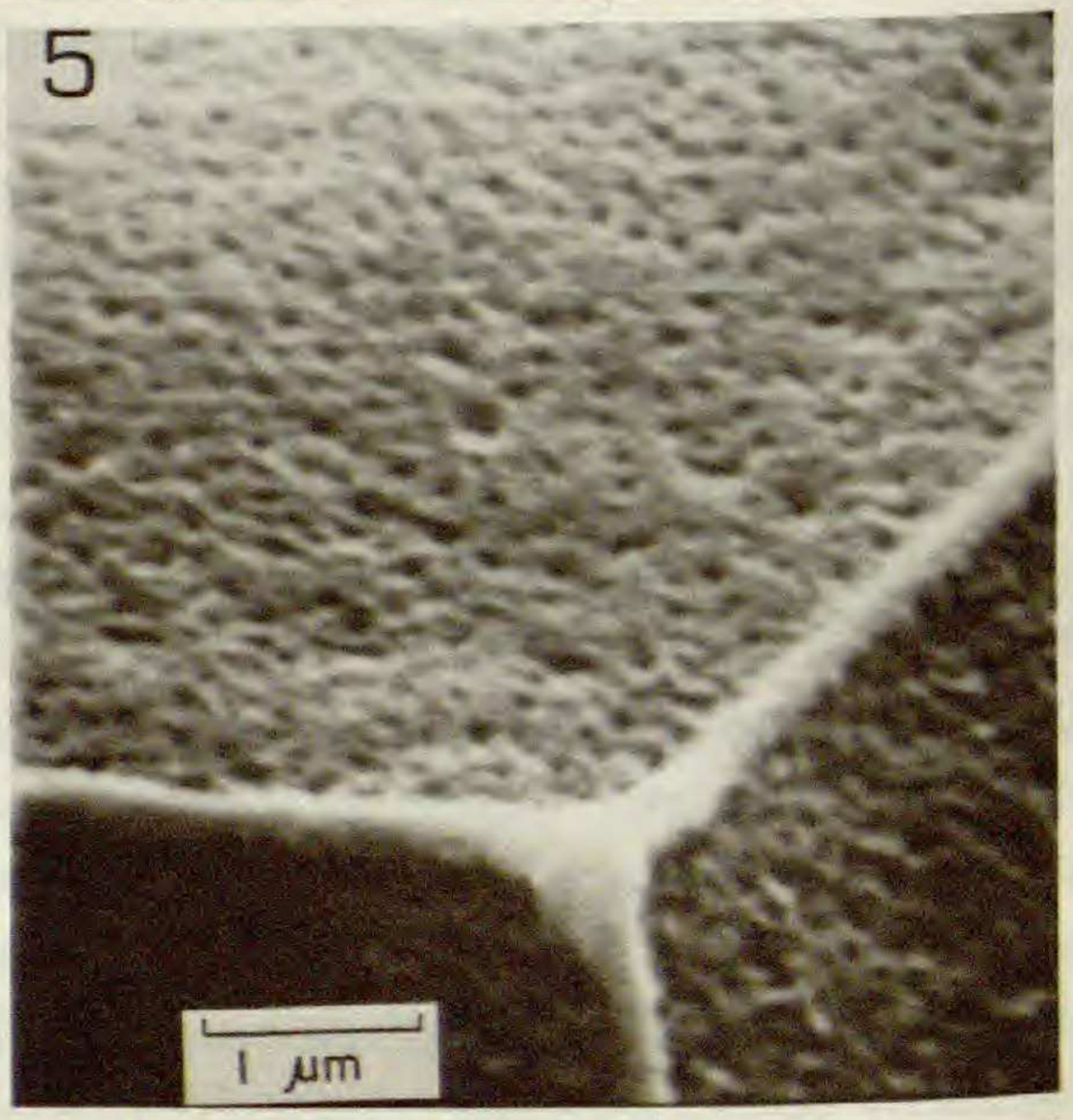
Viability was studied by staining the spores with acetocarmine. Acetocarmine stains the protoplasm and in so doing suggests that the spore is living or viable, but it does not signify that the stained spore can germinate. In this paper the term viable means living as defined by Bewley & Black (1978). For example, immature embryos of *Annona crassiflora* are viable or living, but they do not germinate for at least 200 days after harvest (Rizzini, 1973). All spores tested for viability were from specimens collected in 1982. After breaking the walls mechanically the spores were stained with acetocarmine. The relationship between stained and nonstained spores with broken walls was independent of spore shape. Results are shown in Table 6. In all cases the percentage of stained spores was above 70%. The majority of spores exhibited cellular content when they were subjected to the Wodehouse method (Fig. 3). Thus, all specimens had viable (living) spores, regardless of spore shape.

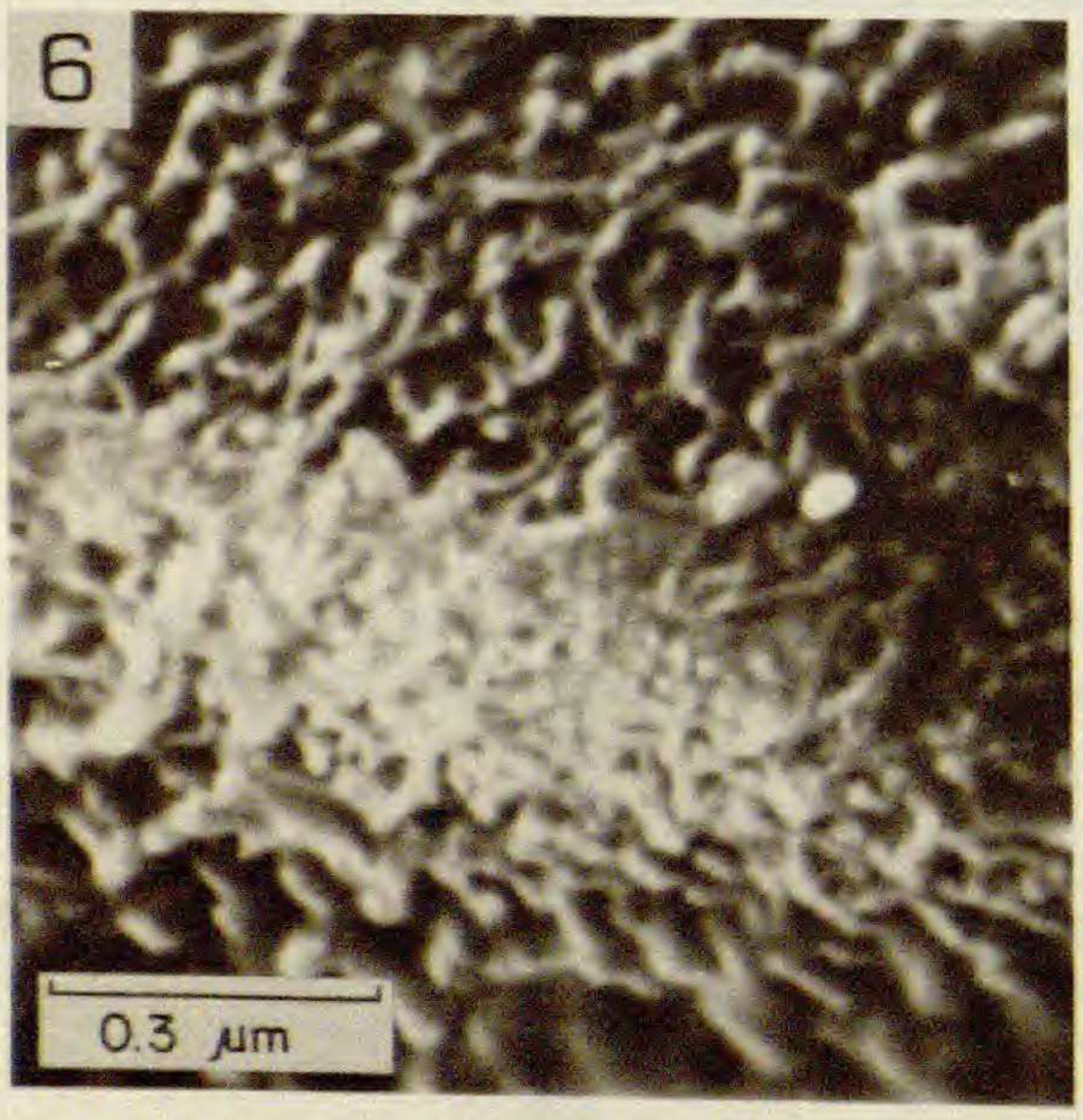
TABLE 6. Viability of Freshly Collected Spores of Five Specimens of *Trichipteris corcovadensis*, Collected in 1982. Spores Were Stained with Acetocarmine After Having Their Walls Broken Mechanically.

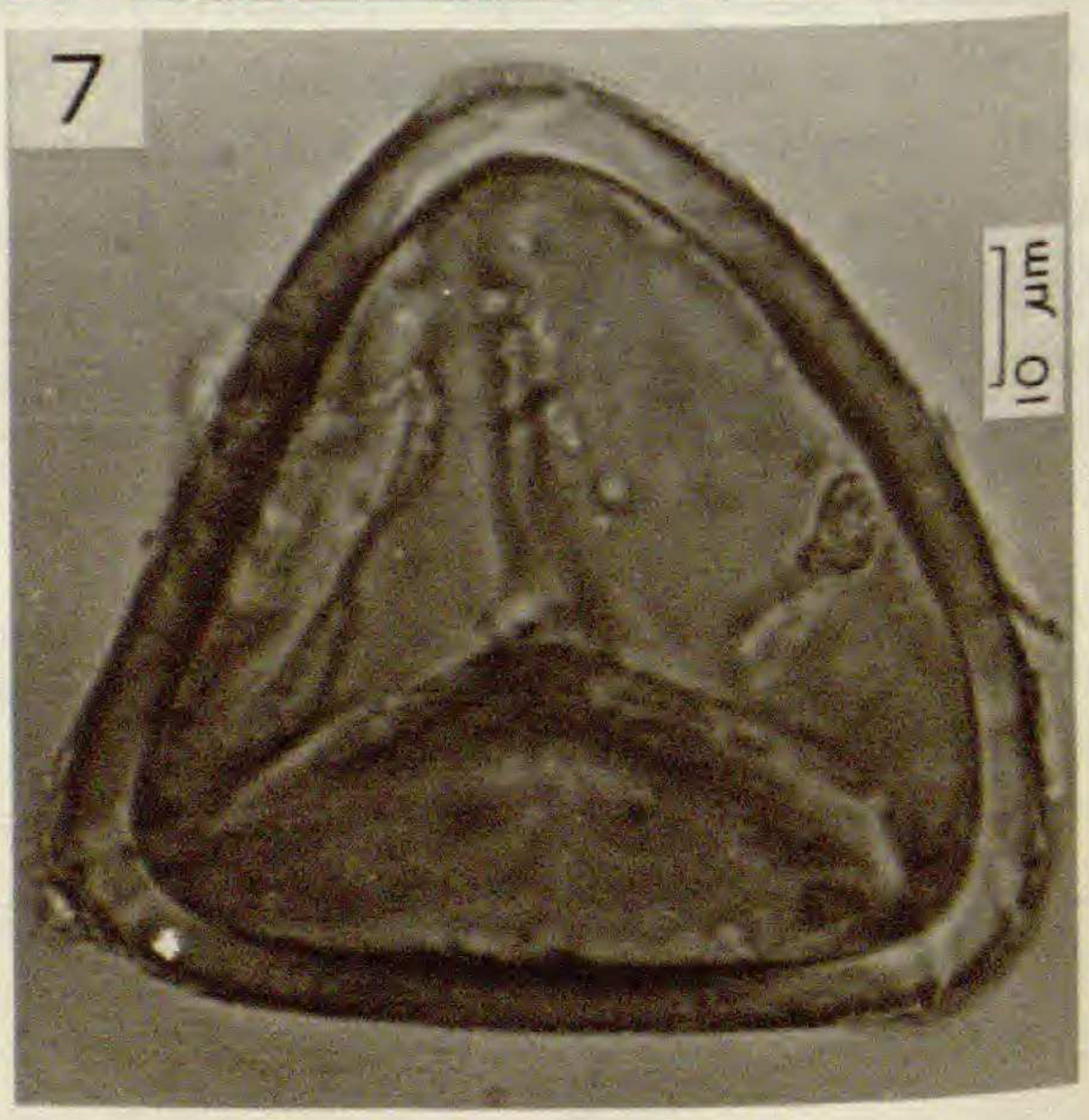
Specimen	Viability (%)
A	88
B	79
C	86
D	77
E	76











FIGS. 3–7. Spores of *Trichipteris*. FIG. 3. Specimen C treated by the Wodehouse method, showing the cellular content. FIG. 4. Surface view of the spore of specimen E, scanning electron photomicro-

TABLE 7. Total Lipid and Soluble Protein in Spores of Five Specimens of *Trichipteris corcovadensis* Collected in Different Years.

Specimen—year	mg·100 mg^{-1} spores	
	Lipid*	Protein*
A—1982	44.2	1.14
B—1981	47.5	1.01
B—1982	48.8	0.93
C—1982	50.1	1.11
C—1983	45.0	—
D—1982	—	0.98
E—1982	—	1.02

* $F_{5\%}$ not significant for lipids and protein.

The content of total lipids and soluble protein was determined in spores collected in different years. About 47% of the weight of the spores is lipid and only 1% is protein (Table 7). No differences could be seen in lipid and protein content in the different specimens studied. Gemmrich (1977, 1982) showed similar values for lipids (over 59% by weight) with spores of *Anemia phyllitidis*. According to Cran (1979), 20% by weight of spores of *Dryopteris pseudomas* is lipid.

Measurements of the equatorial diameter and the trilete length (see Fig. 2A) of spores after acetolysis are shown in Table 8. Spores from specimen A showed the largest diameter, but this diameter was not statistically different from that of spores from specimen B. In all cases the length of the trilete is about 1.6 times smaller than the equatorial diameter independent of the spore.

TABLE 8. Equatorial Diameter and Trilete Length of Five Specimens of *Trichipteris corcovadensis* Collected in Different years.

Specimen—year	Equatorial diameter $\bar{x}$ μm		Trilete length $\bar{x}$ μm	
	Shape I	Shape II	Shape I	Shape II
A—1982	73.1^{a}	—	46.0^{a}	—
B—1980	57.8^{d}	57.0^{d}	35.9^{c}	34.8^{c}
B—1981	71.5^{a}	57.8^{d}	46.3^{a}	45.0^{ab}
B—1982	64.2^{bc}	58.3^{d}	41.6^{b}	37.6^{c}
C—1982	65.5^{b}	57.4^{d}	40.5^{bc}	37.4^{c}
D—1982	68.5^{ab}	61.1^{cd}	43.9^{ab}	37.3^{c}
E—1982	68.5^{ab}	62.3^{c}	44.0^{ab}	37.7^{c}
$LSD_{5\%}$ (Tukey)	3.2		3.6	
Coefficient of variation	5.4%		9.8%	

graph. FIG. 5. Surface view of specimen D, scanning electron photomicrograph. FIG. 6. Surface view of specimen B showing the perine, scanning electron photomicrograph. FIG. 7. Perine of specimen B after treatment with sodium carbonate, as shown in the optical microscope.

TABLE 9. Main Aspects of the Surface of Spores of Five Specimens of *Trichipteris corcovadensis*.

Specimen	Main sculpture	Pits near trilete scar
A	granulate ?	evident
B	granulate ? or pitted, intercalated with small granules	evident
C	psilate, clearly pitted	smaller near the trilete scar
D	psilate, delicately pitted	absent
E	psilate, with small and few pits	evident

The surface of the spores was observed through the optical and the scanning electron microscopes. The sculpture is variable and variations occur not only among the five specimens but also in the same sample. An idea of this variation is given in Table 9, but even so it was possible to define the main sculpture pattern of the spores from each specimen. For example, spores from specimen A show a granulate sculptine while specimen D exhibits a psilate pattern. Specimen E shows the trilate scar demarcated by pits (Fig. 4), which are not observed in spores from specimen D (Fig. 5).

According to Gastony (1974), the perine is lacking in many species of *Trichipteris*. In the present case, perine was observed under both the scanning (Fig. 6) and optical microscopes (Fig. 7). Data presented in Table 10 were obtained with the optical microscope (spores treated with sodium carbonate before acetolysis). It can be seen in Table 10 that 94% of spores of specimen A showed immature granulate perine. Perine at different stages of development was present in spores independent of their shape.

According to Gastony & Tryon (1976) perinous sculptine is attained quite late in spore maturation. They state that "when dried, very immature spores will collapse as the result of incomplete development of mature wall structure and such spores will appear pinched in microscopy". Maturation is a continuum, and even though the mature wall structure will preclude the pinched appearance upon drying, the late development in the final perine is not attained yet. Spores

TABLE 10. Frequency of Spores with Perine of *Trichipteris corcovadensis* Collected in Different Years.

Specimen—year	Perine (%)
A—1982	94[a]
B—1980	79[c]
B—1981	86[b]
B—1982	81[c]
C—1982	74[d]
D—1982	78[cd]
E—1982	67[e]
$LSD_{5\%}$ (Tukey)	4.1

not quite fully mature will present different stages of perine deposition (Gastony & Tryon, 1976). They rely on their experience to judge immature, partially mature, or fully mature perines.

Taking into account the work of Gastony (1979), all spores of specimens B, C, D, E and most of spores from A are immature. None of the spores of specimen A present the perine as hair-like processes, considered by Gastony (1979) as a feature of mature perine, which identifies a mature spore. However, some of the immature spores of specimen A germinate, showing that even though they are not fully mature morphologically, they have reached maturity from a physiological point of view. The majority of spores were viable (i.e., stainable), but only the almost mature ones (morphologically) of specimen A actually germinated.

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SHORTER NOTE

New Locations for *Isoëtes tegetiformans* in Georgia.—Since its discovery in one pool on Heggies Rock, Columbia County, Georgia, in 1976, and its description in 1978 (Rury, Amer. Fern J. 68:99–108), fourteen more pools of *I. tegetiformans* Rury have been found in Columbia, Greene, Hancock, and Putnam Counties, Georgia (Fig. 1). James R. Allison of Lawrenceville, Georgia, discovered these populations at six new sites during 1978 and 1979, while exploring some 150 flatrock exposures in four states.

In May 1978, Dr. James G. Bruce and I visited the first of these new localities (Greene Co.) with Mr. Allison and confirmed the presence of *I. tegetiformans* in five different pools. In a December 1979 letter to me, Mr. Allison reported

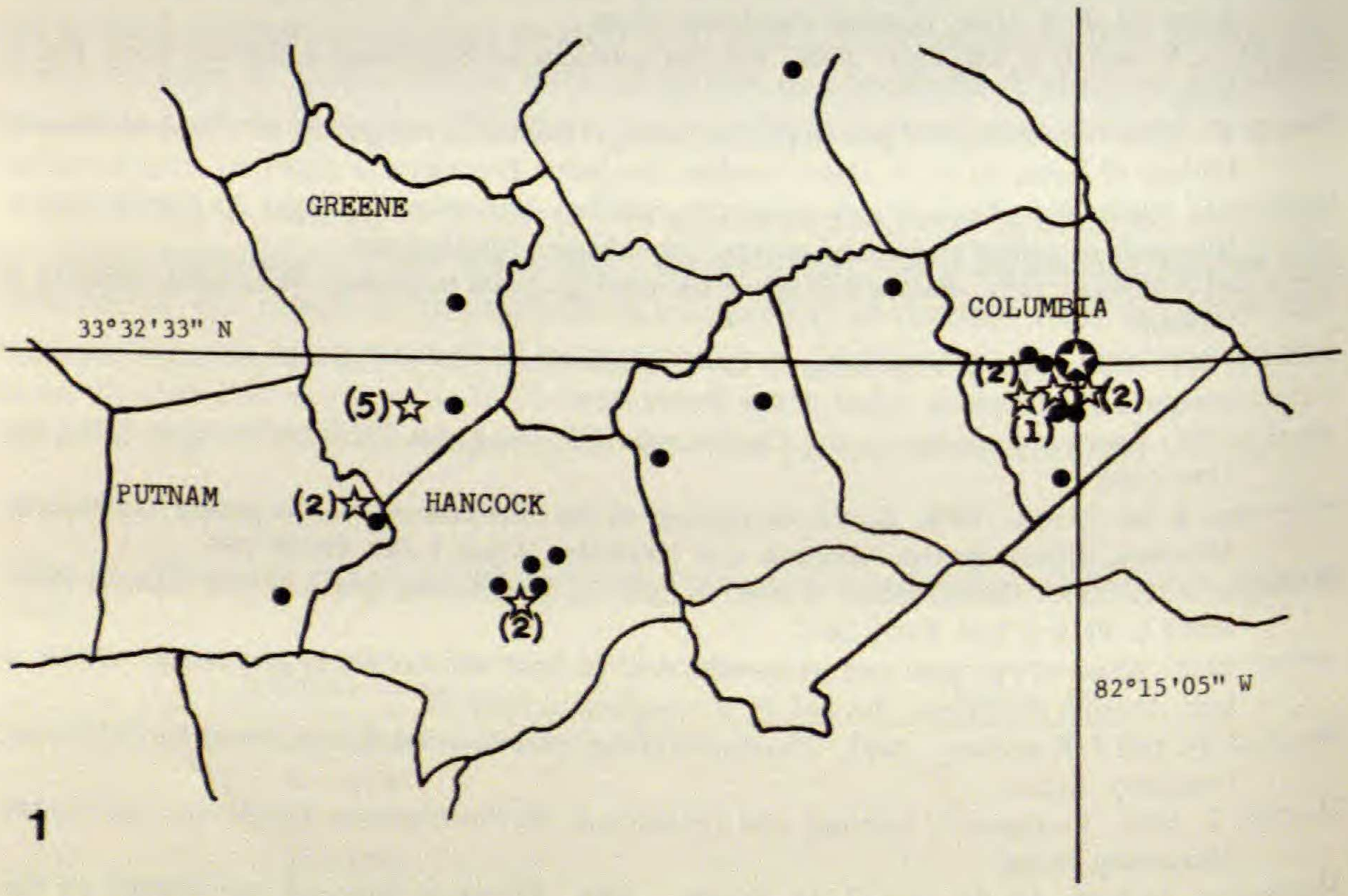

FIG. 1. *Isoëtes tegetiformans* in Georgia. Distribution map modified from J. R. Allison's 1979 letter. Encircled star and associated map coordinates designate Heggies Rock, the type locality. Stars denote new localities; numbers in parentheses indicate the number of populations (pools) found at a given locality. Solid circles denote granite outcrops searched but lacking populations.